Specificity of DNA-associated Nuclear Antigens in HeLa Cells and Distribution during the Cell Cycle

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ABSTRACT

Chromosomal nonhistone protein:DNA complexes prepared from synchronized HeLa cells were used to immunize white rabbits. The antisera reacted specifically in complement fixation tests with chromatin isolated from HeLa cells and not with those from a number of other human and animal tissues. Specificity to this cell type was also demonstrated by immunocytochemical reaction. Both immunocytochemical tests revealed that the specific antigens are continuously present throughout the cell cycle. The immunological activity was dependent upon chromosomal nonhistone protein(s) being bound to DNA. Our findings are consistent with these chromatin antigens being stable nuclear components [complexes of chromosomal nonhistone protein(s) with DNA] characteristic of cellular differentiation.

INTRODUCTION

The presence of chromatin antigens unique to species, tissues, and cell types has been reported by a number of laboratories (3, 8, 9, 12, 13, 18, 31, 32, 41). Tissue- or cell type-specific chromatin antigens were detected most frequently when nonhistone protein:DNA complexes were used for immunization. In these cases, antigenicity was closely dependent upon the binding of nonhistone protein fraction to homologous DNA (9, 10, 32—35). Neither DNA nor chromosomal proteins constituting the immunogen complex when assayed alone demonstrated immunological specificity. These specific chromatin antigens changed during normal differentiation (12, 26) and with the induction of malignant growth (8, 9). The nonhistone protein component(s) of the antigenic complexes was found to represent a small fraction of the total chromosomal protein content of the cell and was of limited heterogeneity (16, 33, 34).

Chromatin antigens of Novikoff hepatoma cells were not detected in chromatin prepared from normal rat liver (7—9, 34, 38). However, cross-reacting antigens were discovered in chromatin isolated from regenerating rat liver, embryonic liver, and in other experimental rat tumor models (7, 38). The antigens in regenerating rat liver were detected only at certain times following partial hepatectomy (7). This restricted time for antigen detection following the induction of parasynchronous cell proliferation suggested that the antigenic complexes of chromosomal nonhistone proteins with DNA in Novikoff hepatoma chromatin may represent cell cycle-specific antigens found in normal cells only during their replication. All of the immunological activity against regenerating rat liver chromatin was eliminated by absorbing the antiserum with chromatin prepared from regenerating rat liver, but the activity against Novikoff chromatin antigens was only partially removed, thus suggesting that Novikoff-specific chromatin antigens may originate from ones that are common to proliferating liver cells and also from some that are unique to cancer. Since the antiserum to the Novikoff chromatin antigen(s) reacted with chromatin isolated from fetal liver, it further suggested a similarity in the expression of chromatin antigens specific for malignant cells to other tumor-associated antigens or oncodevelopmental gene products. There is evidence that the amount of some tumor-associated antigens (4) and the H-2 Moloney leukemia virus-determined cell surface antigens (14) are cell cycle dependent, as would also be expected for proteins involved in replication of the genome (reviewed in Ref. 37).

Clarification of the relationship between the expression of the specific antigenic complexes of nonhistone proteins with DNA and cellular proliferation required the use of a rapidly growing in vitro model system whose synchrony could be easily manipulated. Using HeLa cells, we were able to demonstrate once again the surprising cell type specificity of this group of chromatin antigens. However, their levels detected during cellular replication did not change measurably.

MATERIALS AND METHODS

HeLa S3 cells were maintained in suspension cultures containing Joklik-modified Eagle's minimum essential medium supplemented with 3.5% each of calf and fetal calf serum. Cells were routinely synchronized by double dThd block, and in special cases, they were treated with either 1-β-D-arabinofuranosylcytosine (40 μg/ml) or hydroxyurea (10 mM). The S-phase cells were harvested 3 hr after release from the second thymidine block, and the G2 cells were harvested 8 hr after release. Cells in the G1 phase were obtained 2 hr after selective detachment of mitotic cells. The level of cell synchrony was monitored by determination of mitotic index and autoradiographic determination of [3H]dThd incorporation. Cells were labeled for 30 min in [3H]dThd (1 μCi/ml; 50 to 60 Ci/mmol).

Nuclei were prepared from synchronized or logarithmically growing cells by use of the non-ionic detergent Triton X-100 (27). Cells were harvested, washed 3 times with 80 volumes of spinner salts (Grand Island Biological Co.), and lysed with 80 volumes of 80 mM NaCl:20 mM EDTA:1% Triton X-100 (pH 7.2). Nuclei were pelleted by centrifugation at 1000 × g for 3 min, washed 2 times in 0.15 M NaCl:10 mM Tris (pH 8.0), and collected by centrifugation at 1000 × g for 3 min. Nuclei isolated in this manner were free of cytoplasmic contamination when observed by phase-contrast microscopy. Nuclei were
lysed in 60 volumes of double-distilled water by gentle homogenization. The chromatin was allowed to swell in an ice bath for 20 min and was then pelleted by centrifugation at 12,000 x g for 20 min. Chromatin concentration was adjusted for complement fixation tests, digestion, absorption, or use as an immunogen.

Micrococcal nuclease (Worthington Biochemical Corp., Freehold, N. J.) was incubated with total chromatin in 40 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1.0 mM CaCl₂, and 0.1 mM phenylmethylsulfon fluoride for 30 min (37°C) at the concentrations indicated. The reaction was stopped by the addition of perchloric acid to 0.8 N and was allowed to stand in ice for 15 min. The absorbance of the supernatants at 260 nm was obtained following centrifugation (1500 x g; 20 min). After correcting for hyperchromic effect, these readings were used to determine the amount of acid-soluble DNA.

Antisera to S and G2 cells showed complement-fixing activity only at dilutions of 1:200 or greater when tested with their respective chromatin preparations. Serum collected from these 2 animals prior to immunization gave no complement-fixing activity when tested at the same dilutions. The second antisera to S and one from G2-synchronized cells gave complement-fixing activities in complement-fixation tests, digestion, absorption, or use as an immunogen.

RESULTS

Dehistonized chromatin was prepared from G1-, S-, and G2-synchronized HeLa cells, and each preparation was used to synthesize 2 rabbits. One antiserum to chromatin isolated from S and one from G2-synchronized cells gave complement-fixing activity at dilutions of 1:200 or greater when tested with their respective chromatin preparations. Serum collected from these 2 animals prior to immunization gave no complement-fixing activity when tested at the same dilutions. The second antisera to chromatin from S and G2 cells as well as both antisera to G1 cells showed complement-fixing activity only at dilutions of 1:50 and lower. The antisera to chromatin from G1-synchronized cells showed the same low level of activity when reacted with chromatin from cells synchronized in any of the 3 phases of the cycle or with material prepared from logarithmically growing HeLa cells. However, due to technical problems to be discussed later, these low-titer antisera were not further tested for specificity of reaction.

Specificity of the 2 higher titer antisera to chromatin from S- and G2-synchronized cells was demonstrated by reacting antisera with chromatins prepared from other normal and malignant human tissues and tissue culture line as well as normal and malignant rat tissues (Chart 1). As can be seen in this chart, only HeLa chromatin (log-phase cells) fixed the complement in the presence of HeLa antiserum. To determine if either antiserum reacted preferentially with chromatin during one phase of the cell cycle, batches of 3 chromatins were prepared for testing, one from each of G1-, S-, and G2-synchronized HeLa cells. Greater than 98% of S-phase cells were in DNA synthesis. Between 70 and 80% of cells harvested 8 hr after release from G1/S block were in G2 phase. More than 95% of the cells were in G1 phase 2 hr after selective detachment of mitotic cells. Five batches of chromatin isolated from G1-, S-, and G2-synchronized cells were tested by complement fixation.

The 2 antisera showed a slight preference for G1, chromatin in the complement-fixation assays, although they were raised to chromatin prepared from S- and G2-synchronized cells. Both antisera exhibited the same maximal level of reaction with each of the 3 chromatins within the batch. However, in subsequent batches of chromatins from the 3 phases of the cell cycle, the S- and G2-derived materials on occasion also showed slight preferential activity with the antisera than did the other 2 phases. No chromatin preparation from either of the 3 phases (G1, G2, and S) consistently gave highest, lowest, or middle reactivities in complement-fixation tests with either antiserum, but antisera to S- and G2-phase chromatins gave the same relative order of reactivity within any one batch of chromatins from the 3 phases of the cycle. Also, each antiserum always showed the same maximal complement-fixing activities with chromatins from all 3 phases. A representative complement-fixation reaction with one of the antisera, anti-G2, reacted with...
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One batch of chromatin is shown in Chart 2 (the closed symbols) where a slight preferential reactivity with S-phase chromatin can be seen.

Antiserum to dehistonized chromatin from G2-synchronized HeLa cells was used to determine if chromatin from one phase of the cell cycle could be used to absorb out reacting antibody against chromatin preparations from all 3 phases. The antiserum was initially reacted with a batch of chromatin from the 3 phases (G1, S, and G2) at varying dilution of IgG fraction (Chart 2). Absorbing the antibody with G2 chromatin in a ratio of 300 μg of DNA per ml of a 1:50 dilution of antibody caused the elimination of the complement fixation reactivity of the antibody with all 3 chromatins (Chart 2; the open symbols). This ratio of chromatin as DNA to antibody dilution was about one and one-half times the amount in the complement-fixation test which gave maximal activity (Chart 2). Use of G1 chromatin in a ratio of one-half the amount needed to reach maximal complement-fixation activity also resulted in the elimination of activity against all 3 chromatins (data not shown). Absorbing the G2 antibody with a nonreacting chromatin, rat liver, (Chart 1) had no effect on the complement-fixing activity of the antiserum reacted with HeLa chromatin preparations.

In another approach, a finer subdivision of S-phase was obtained by harvesting cells at hourly intervals after release from the second thymidine block of logarithmically growing HeLa cells. The chromatins prepared from cells harvested from 1 to 8 hr after release showed the same maximal complement-fixation activity. Antisera also reacted readily with isolated metaphase chromosomes. In view of their highly condensed conformation, such results indicate that antigen availability per se is unlikely to be a major consideration in cell cycle dependence.

Consistent with the lack of cell cycle specificity, the antisera gave uniform immunocytochemical staining reaction with all cells in a population of logarithmically growing HeLa cells (Fig. 1) where autoradiographic analysis of [3H]dThd incorporation from a 30-min exposure showed an average of 38% labeled nuclei in these cultures. No immunocytochemical staining reaction was seen when the antiserum was tested on WI-38 cells or with WI-38 cells transformed by SV40 virus (Fig. 2). No positive staining reaction was observed with cells in normal peripheral blood smears or with any of the cells present in frozen tissue sections of specimens of squamous cell carcinoma, in epidermoid cancer of the lung, or in uterine cancer (data not shown).

The effects of inhibitors of DNA synthesis on immunological activity were also tested. S- and G2-phase cells were harvested after treatment for 30 min with 1-β-d-arabinofuranosycytosine or hydroxyurea. The same absolute maximal amount of complement fixation activity was obtained with either antiserum when chromatins from the inhibited cells were reacted at the same time as chromatins from either G1-synchronized cells or exponentially growing HeLa cells.

The effect of cell density was determined by testing chromatins prepared from cells harvested at different densities during logarithmic growth (Chart 3). The chromatins gave the same maximal reactivity with some lateral shifts in the reaction curves without direct correlation to density.

When chromat from HeLa cells was treated with increasing amounts of micrococcal nuclease, immunological activity (complement-fixation) was progressively eliminated until no reaction was detected (Chart 4). A precipitate was observed early in the reaction at each level of nuclease, and the amount of acid-soluble DNA obtained ranged from 44% at 50 units to 48% at 2000 units of enzyme.

Fig. 1. Immunocytochemical staining reaction of antiserum (1:200) to nonhistone protein:DNA complexes isolated from G2-synchronized cells with logarithmically growing HeLa cells (a). Immunocytochemical-staining reaction with serum (1:200) collected prior to immunization with logarithmically growing HeLa cells (b). x 250.
DISCUSSION

Complement-fixation tests and immunocytochemical-staining reactions revealed the presence of chromatin antigens specific for HeLa cells. One antiserum to chromatin from G2 and one to S-phase-synchronized HeLa cells showed the same immunological specificity for chromatin derived from HeLa as well as for the preferential reactivities within batches of chromatin from 3 phases of the HeLa cell cycle (G1, S, and G2) including metaphase chromosomes. The similarity in the reactions of the 2 antisera demonstrate that antigens also serving as immunogens in S and G2 chromatin preparations are the same. The 2 antisera to chromatin from G2-synchronized cells showed the same low complement-fixing activity with chromatin isolated from G1-, S-, and G2-synchronized cells. However, weakly reacting antisera must be used in low dilution where the antisera usually contain complement-inactivating activity which adversely affects the detection of immunological activity. The unpredictable nature of this activity prevents the routine use of these antisera. Therefore, the antiserum to chromatin from G1 cells were not tested for species and cell type specificity. Our inability to obtain more reactive antiserum with G1-derived chromatin may indicate that G1 chromatin is immunogenically deficient. It is also possible that the lower activity of antisera obtained from G1 chromatin and the low activity of antisera obtained from the second animal immunized with G2 and one from S-phase chromatin reflect a variability in response of animals to immunization.

In contrast to immunogenic potential, the presence of antigen during the cell cycle can be generalized to all phases including G1. Chromatin preparations from synchronized HeLa cells (G1, S, or G2) and that prepared from logarithmically growing cells showed the same levels of complement-fixing activity when tested with the antisera to S and G2 phases in 10 separate immunological assays. Furthermore, the immunological activity of antiserum to chromatin from the 3 phases of the cycle could be eliminated by absorbing with chromatin prepared from one phase as demonstrated with chromatin from G1 and G2 cells. Immunocytochemical analysis of logarithmically growing HeLa cells confirmed these results and showed a uniform staining reaction in all cells of the population. Positive staining of only a fraction of the cells in the rapidly proliferating population would suggest restricted antigen presence during the cell cycle. Since this was not observed, all of the results demonstrate that chromosomal nonhistone protein:DNA antigen com-
plexes specific for HeLa cells detected with antisera to S and G2 cells are continuously present throughout the cycle of the replicating HeLa cells.

Because of the antigens sensitivity to chemical fixatives, none was used prior to the localization by immunocytochemistry. Therefore, the uniform staining reaction was interpreted as evidence for the invariant amount of antigen present throughout the cycle and not as a rigid demonstration of its intracellular localization. Although the reaction is centered over the nucleus, the questionable effectiveness of freeze-drying in fixing the antigen makes it possible that some may have diffused out of this compartment. Also, it is reasonable to expect to find some antigen in the cytoplasm while being synthesized in replicating cells in order to maintain the constant amount found in chromatin. However, considering the antigens requirement for DNA to exhibit immunological activity in the complement-fixation assay, it is doubtful that antigen in the cytoplasm would be detected by the localization method, and any reaction there may be the result of diffusion. In any case, the uniform staining of all log-phase cells is consistent with the invariant amount of antigen in cycling cells or at least that amount bound to DNA.

The slight lateral shifting in the activity curves observed within batches of cell cycle-phase chromatin (Chart 2) were shown to be independent of phase and density (Chart 3). It might be assumed that they arise from variable growth conditions or treatment effects during chromatin preparation. This lateral displacement could be due to differences in amounts of antigen relative to DNA content or to conformational changes affecting antibody-antigen aggregate characteristics which determine the extent of complement fixation (19). However, the major reacting antigen(s) is most likely present in all cases; as the same maximal complement-fixing activity is always achieved as additional antigen is reacted. General conformational changes can lead to lateral shifting in complement-fixation curves (19). Greater changes in the antigen resulting in the loss of some reacting sites caused a vertical shift, as seen in Chart 4, where nuclease digestion eliminated the immunological activity. As can be seen in this figure, maximal complement-fixing activity could not be restored by increasing the concentration of digested antigen; an observation consistent with the loss of reacting antigenic components after staphylococcal nuclease treatment.

Progressive loss of immunological activity with increasing amounts of nuclease did not coincide with the appearance of a precipitate during the digestion. A similar amount of precipitate appeared at all levels of nuclease even where immunological activity was not diminished. Similarly, the extent of DNA digested (acid soluble) noted with increased levels of nuclease does not show dramatic change where immunological activity is affected. This suggests that a small amount of DNA protected to some extent from nuclease digestion is essential for immunological activity. The significance of this reaction was investigated in more detail in a separate study (5, 6). After limited nuclease digestion, the immunological activity was found in the high-molecular weight oligonucleosome fraction and not with the monomers. In additional experiments with metaphase chromosomes, the antigens were found to be directed to the chromosome scaffold proteins (5). The scaffold was initially described as the basic structural component of the chromosome (1). However, the possibility of a strict cell-type specificity due either to the proteins present or to their modification state is suggestive of an additional role.

A number of studies have shown activity, modification, and quantitative changes in the chromosomal nonhistone proteins occurring during the cell cycle coincident with rapid changes in transcriptional activity. Nonhistone proteins isolated from cells synchronized in various phases of the cell cycle differentially affect in vitro RNA transcription (23). By contrast, the nonhistone proteins show very little qualitative change in synchronously dividing HeLa cells when analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gels (2, 17). Although the total quantity of nonhistone protein changed markedly, it seemed to vary as a whole (17). Our immunological findings are in accord with the lack of any qualitative change in nonhistone protein during the cell cycle. However, they may not be consistent with the uniform quantitative change in these proteins observed relative to DNA content during replication (17). In addition, the major nonhistone protein species detected by one-dimensional gel electrophoresis are known to be common constituents of chromatin in many tissues and cell types (15, 25). Applying high-resolution, 2-dimensional gel electrophoretic methods to chromatin protein analysis has more thoroughly resolved the heterogeneity of the polypeptides found in chromatin and has also shown the great similarity in the major components in different normal tissues and cells and between normal and transformed cells (20, 29, 39). The strict specificity of the HeLa antisera to an individual cell type, as indicated in Chart 1 and demonstrated in another study with these antisera (5), would make it unlikely that the antigens being detected are the major components common to most cells of an organism. However, these major polypeptide components of chromatin could be antigenically unique due to modification or organization when complexed with DNA. They may also be recognizing unique minor components specific for cell type. Some minor components of restricted distribution have been detected by the 2-dimensional gel analysis (20, 29, 39). The present results do not indicate which of the above alternatives is correct, therefore it is not possible to equate the HeLa chromosomal nonhistone protein antigens to specific chromosomal polypeptides detected by electrophoretic analysis.

Since our immunogen preparation is heterogeneous, it is possible that some components could vary or undergo minor conformational changes during the cell cycle but go undetected due to the presence of a single or a large number of noncycle-specific components. However, the results show that these overshadowing determinants must be cell type specific. Our previous experience with antisera raised in a similar manner, as stated in the introduction, has been that only a limited number of polypeptides, separated by sodium dodecyl sulfate-acrylamide gel electrophoresis, actually serve as antigens. Apparently, the treatment of chromatin with high salt and urea renders many nonhistone protein components immunologically inactive. Some chromosomal protein antigens are known to be sensitive to urea treatment (40). It appears likely that our preparation of immunogen results in the selection of a specific group of antigens, which are closely associated with DNA, are not cell cycle dependent and show a highly specific cell type distribution.

The present results demonstrate that the major HeLa-specific, DNA-associated antigenic components are not cell cycle specific and therefore are not related to growth-associated
nuclear antigens which have been recently found as common features of replicating human cells (21, 24). Similarly, they are not related to a species-specific, cell cycle-dependent antigen detected in human cells by Tsutsui et al. (30). Immunocytochemical detection of that antigen in resting cells (G0) and following the induction of proliferation showed a greater number of positive-reacting nuclei in the resting state. Once induced to proliferate, the level of positive-staining nuclei declined and was not affected by the progression of the cells through the cycle. The HeLa chromatin antigens are more restricted in their distribution and are continuously detected during the cell cycle. The presence of a variety of chromatin antigens probably accounts for the divergent reports of specificity for species, tissues, and cell type (3, 9, 12, 13, 18, 22, 30–32, 38, 41). The choice of method for immunogen preparation varied in these studies and could be an important factor in determining which chromatin antigens will elicit the immunological response. The questions surrounding the distributions of chromatin antigens, their heterogeneity, and the possible relationship to existing known chromatin polypeptide components will be clarified only when more of the antigens are isolated and characterized.

The experimental data presented show that antisera were raised to cell type specific chromatin antigen(s), that the immunoactivity of the antisera was dependent on the binding of the antigenic protein to its DNA, and that similar amounts of antigen were detected relative to the quantity of DNA throughout the cell cycle. The cell cycle stability of the specific antigen(s) reported herein is in line with its being a unique and fundamental cellular characteristic determined by the state of differentiation.

ACKNOWLEDGMENTS
We gratefully acknowledge the excellent technical assistance of Beverly Bell, and we wish to thank Dr. Frank Chytil and Mrs. Lucy Chytil for their help in raising antisera.

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